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Breast cancer detection and diagnosis has been limited by the lack of early and accurate markers of disease. Efforts to date have largely relied on insensitive measures such as mammography or breast examination for detection, with conventional histopathology for diagnosis of breast cancer. The goal of this project is to utilize new molecular diagnostic techniques to improve breast cancer diagnosis. Towards this goal we evaluated the frequency of genetic alterations in variable short tandem repeats (microsatellites) in microdissected breast tissue including normal, in situ carcinoma, invasive, and metastatic carcinoma from the same breast lesion. On the same tissues, in parallel, we are testing the utility of a novel marker, the enzyme telomerase. In this way the prognostic and genetic changes that occur within a single breast lesion can be determined. The long range goal of this project is to develop molecular diagnostic techniques which may be used in the detection of breast cancer and to utilize these techniques in defining the genetic alterations associated with neoplastic progression in breast.

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INTRODUCTION

Subject, purpose and scope of research:

Breast cancer screening and diagnosis have relied on conventional histopathology and clinical parameters, which are often insensitive. Newly developed molecular diagnostic techniques such as detection of genetic alterations in microsatellite markers have been used to determine clonal mutations in a variety of tumors and promise to be more sensitive measures of disease extent. We hypothesize that these techniques will allow more precise definition of the genetic progression of breast neoplasia and improve the diagnosis of breast cancer.

To test the central hypothesis that molecular diagnostic techniques can be developed to aid in the detection of breast cancer, the following specific aims were proposed:

Specific Aim 1. To characterize the frequency of microsatellite alterations in breast cancer and to develop informative panels of microsatellite markers in breast cancer.

We will examine, in microdissected normal and neoplastic breast tissue, a series of microsatellites with documented genetic alterations in human neoplasia. We hypothesize that a majority of breast cancers can be detected by using a panel of markers.

Specific Aim 2. To apply such a panel of microsatellite markers to microdissected breast tissue including normal, hyperplasia, *in situ* carcinoma, invasive, and metastatic carcinoma from the same breast.

In view of our recent findings on the infrequency of microsatellite expansion in many markers in sporadic breast cancer, we have added another task to the 1st and 2nd specific aims, using a marker that was developed after this grant was approved. We

examined and ascertained that telomerase is a good marker for breast cancer. Next we will evaluate the utility of telomerase as an early marker of breast cancer in the same tissues that are undergoing analysis for loss of heterozygosity (LOH) in Specific Aim 2.

Our goal is to employ variable short tandem-repeat DNA sequences (microsatellites) to determine loss of heterozygosity and the presence of telomerase activity in microdissected tissue samples to improve the diagnosis and prognosis of breast cancer.

Background of previous work:

Recent molecular techniques which are being studied in the detection of malignancy include the use of tumor-specific probes. Unfortunately, at this time, there are no known pathognomonic mutations in breast cancer. For detection in the absence of a known specific mutation, other techniques are needed. One approach is based on short tandem-repeat DNA sequences (microsatellites)[1--5]. Microsatellites are highly variable sequences commonly found throughout the human genome. Microsatellite alterations include imbalance, in which the allele is of the same size, but of greater or lesser intensity than the corresponding nomal allele, or instability, in which an allele of novel size is found in the neoplastic tissue[1--5].

Microsatellite imbalance is generally thought to result from deletion or amplification of the repeat sequence, and is commonly used as a marker of potential tumor suppressor genes. Such imbalances have been identified in numerous chromosomal sites in breast cancer, including the areas known to contain genes implicated in breast cancer such as erb B-2, BRCA-1, and nm23. Studies employing various microsatellite markers within a specific chromosomal area are proving to be a valuable

tool in identifying areas of potential tumor suppressor gene loss. Several of these recent studies have identified microsatellites which are not only heterozygous in the majority of individuals studied, but evidence allelic imbalance in the majority of tumors studied[1-5].

Another important milestone in breast cancer research is the realization that the variability in results obtained in different laboratories on oncogene and tumor suppressor gene as well as loss of heterozygosity (LOH) analysis are due in large part to the inherent heterogeneity of breast tumors. Their composition varies widely with respect to the stromal/ epithelial compartment as well as to the presence of both benign and malignant lesions in close apposition. The ability to analyze genetic changes in each of the cell compartments has been made possible, firstly by sensitive PCR techniques and secondly, by the ease with which microdissection of sectioned breast tissue slices can now be performed. In fact, using these techniques, investigators have shown the loss of heterozygosity of markers on 3p, 11q, 13q, 16q, 17 p, and 17q in a significant proportion of breast tumors[4, 6, 7]. In addition, they showed that the same loss had taken place in DCIS in the same section of the tissue, thereby providing evidence that the LOH had taken place very early in malignant progression[4, 6, 7]. Such markers show great promise and may prove efficacious in identifying the hyperplasias and/or DCIS that will progress to malignancies versus those that will not.

One of the most exciting recent findings with special importance to breast cancer is the specific and frequent presence of the enzyme telomerase in cancer cells. Telomerase is a ribonucleoprotein enzyme active in germ cells, immortal cell lines, and the majority of malignancies examined, but inactive in most normal somatic tissues. It has been proposed as a potentially useful marker of malignancy. In several studies of primary invasive breast

cancers, approximately 75-95% of tumors demonstrate telomerase activity, while 5-25% are telomerase-negative[9-13]. Obviously, telomerase activity as a marker of invasive breast cancer is more useful if it detects 95% of the tumors rather than 75%, so the determination of the real telomerase-negative proportion of invasive breast cancers becomes crucial. In an exhaustive study of 105 breast tumors, we have determined that close to 98% of breast tumors are positive for telomerase, while normal epithelium from the same breast is not (Appendix 1). We have also found that some DCIS adjacent to the carcinoma are positive for telomerase while others are not (our unpublished observations). These findings point to the potential of telomerase, along with LOH studies, to provide crucial information regarding the biology and genetics of early lesions that may or may not progress to full malignancies.

BODY

In the last one year of this project, we have accomplished the goals of Specific Aim 1, which was to select microsatellite markers to apply to a panel of breast cancers in Specific Aim 2. We have also established telomerase as a promising new marker of breast cancer, and will apply this marker in parallel. During 1997-98, we will address Specific Aim 2 which applies this selected panel of markers to microdissected breast tissue to determine the progression of genetic alterations in normal, premalignant, and malignant tissue. In the long term, the method will hopefully provide a targeted, noninvasive, and more accurate alternative to punch biopsies.

Progress Report:

1. ***Specific Aim 1A.*** To characterize the frequency of genetic alterations in microsatellites in breast cancer and to develop a panel of informative microsatellite markers.

Rationale: Microsatellite alterations represent a promising technique in diagnosis and primary prevention of breast cancer, since they do not require knowledge of the specific mutation, only knowledge of areas frequently altered in breast cancer and their corresponding microsatellites.

Design: Predictive value analysis for new diagnostic method and selection of panel of markers.

Tissue. Approximately 40 breast cancer cases were identified and selected from banked, frozen tissue in the Breast Cancer Program laboratory. These specimens are from mastectomy or excision of a primary breast cancer. Normal and neoplastic tissue will be microdissected, and DNA from normal and neoplastic areas will be amplified via PCR.

Genetic markers: Genetic imbalance in microsatellites was defined as a change of greater than 50% intensity between the normal and neoplastic tissue. Genetic instability in microsatellites is characterized by an increase or decrease in the number of repeated elements and is detected by the presence of novel electrophoretic bands in neoplastic compared to the normal tissue. Any novel band will be considered abnormal and indicative of instability.

Work completed: A prescreen of DNA from 40 breast tumors has been performed as those providing the greatest likelihood of identifying microsatellite alteration.

The following markers were screened based upon the relatively high frequency (more than 50%) of LOH seen at these loci:

Chromosome

3p: D3S2397, D3S1597, EABMD, D3S1244

6q: D6S292, D6S311, D6S310, D6S473, and D6S255,

9q21: D9S171, D9S1748, D9S1749, D9S1751, and D9S1752

11q13-23: Int2, D11S29, D11S35, and D11S528

13q12-14: D13S260, D13S263, and D13S155

16q12-24: D16S541, D16S415, D16S265, and D16S402

17p: D17S513, CHRNB1, TP53, D17S786, and D17S122

17q12-24: THRA1, D17S579, and D17S588

PCR reactions typically contained 1 ul lysate prepared from microdissected cells (20 cell equivalent)[4]. PCR products were then separated using denaturing gel electrophoresis,

and allelic loss was determined by at least a 75% reduction in the relative intensity of one allele in the tumor compared to normal after autoradiography. Allelic loss was usually confirmed by observation of LOH at multiple informative markers mapped to the same chromosomal region. When only one informative marker was recognized for a chromosomal region in a particular tissue sample, LOH was confirmed by repeating the PCR amplification of that marker.

Data analysis and conclusion. The group of markers with the greatest likelihood of being abnormal are two at 3p24, two at 11p15.5 and two at 17p and 17q. We believe that these will provide a reliable and sensitive means to identify tumors, study their clonality, and to study tumor progression in Specific Aim 2. Considering the small amount of DNA obtained from each sample, limiting the number of markers will, we believe, provide optimal chances of success. We observed microsatellite instability very infrequently and not reproducibly at the same locus. These results lead us to conclude that instability in dinucleotide repeat sequences are not common in sporadic breast cancer.

2. ***Specific aim 1B:*** To validate telomerase activity as a potential marker for breast cancer:

Rationale: The reverse transcriptase telomerase is reactivated in breast tumor cells. Therefore, telomerase could prove to be a marker that can distinguish between normal, preneoplastic and malignant breast cells.

Design: Predictive value analysis for new diagnostic method.

Tissue. Approximately 105 breast cancer cases were identified and selected from banked, frozen tissue in the Breast Cancer Program laboratory. These specimens are from mastectomy or excision of a primary breast cancer. Normal and neoplastic tissue were used before and after microdissection.

Work Completed: In the last funding year, we examined 105 breast tumors for telomerase activity on lysates prepared from: whole tissue, 1 frozen section after histopath examination, or microdissected tumor material. We ascertained that close to 97% of breast tumors are positive for telomerase, while 17 microdissected normal lobules were negative for telomerase. Histopathological examination was found to be crucial prior to assaying (see appendix 1). Recent studies have revealed that very weak telomerase activity is detected in some fibroadenomas, and in normal breast tissue during the luteal stage. Activity in tumors is significantly higher than in these tissues. We have also completed a study examining telomerase activity to disease outcome in 300 patients with five or more years of followup (Carey and Sukumar, unpublished observations), the results of which are currently being analyzed. Thus, detection of telomerase activity shows the sensitivity and specificity necessary for a tumor marker.

Methods:

Microdissection and DNA extraction[6, 7]. This technique reduces the heterogeneity of DNA obtained by determining the cells of interest, then selectively removing only those cells under direct light microscope visualization. The selected DNA can then be amplified by PCR.

Unstained frozen (-80°C) tissue will be cut into two 10μm sections by cryotome and placed on 0.1% poly-L-lysine-coated acetate slides. One slide from each tumor will undergo hematoxylin and eosin staining, while the other will remain frozen and unstained.

Cells of interest will be selected from the hematoxylin and eosin-stained slide, then the same area will be chosen from the unstained section. Using a 30-gauge disposable needle under direct light visualization on an Olympus dissecting microscope, the chosen areas will be removed by microdissection. Approximately 50 cells are removed from each area of interest for DNA extraction.

The microdissected cells are immediately placed in 30 µl DNA extraction solution containing 0.01 M Tris-HCl, 0.1 M ethylene diamine tetraacetic acid, pH 8.0, 1% Tween-20, and proteinase K 0.1 mg/ml. The solutions are incubated at 56°C for 3 hours or 37°C overnight. The microcentrifuge tube is then boiled for 5 -7 minutes to inactivate the proteinase K and will be stored at -20°C. Each PCR reaction will use 1µl of this solution.

Microsatellite analysis. DNA is procured by microdissection from paired neoplastic tissue and nonneoplastic tissue from the surrounding breast. Microsatellite sense and antisense primers for each locus of interest will be synthesized by the Johns Hopkins Core Facility. Radiolabelling will be performed by the random oligonucleotide priming method. Both the normal and tumor DNA will undergo approximately 30 cycles of PCR according to conditions determined by the primers used followed by electrophoresis on denaturing gel and autoradiography. Microsatellite alterations will be detected by the presence of allelic imbalance or novel bands present in the neoplastic DNA compared to the nonneoplastic tissue from the same patient.

Polymerase chain reaction (PCR). One microliter of the DNA extraction mixture will be used for PCR. The DNA will undergo approximately 30 cycles of PCR in an Ericomp TwinBlock System Thermocycler under the conditions specified by the primers. PCR will be performed in 12.5 µl aliquots containing PCR buffer, *Taq* polymerase, unlabelled

deoxynucleotides, and [^{32}-P]dCTP (Amersham Corp., Arlington Heights, IL). PCR products will be separated by electrophoresis in denaturing 8% urea-polyacrylamide-formamide gels followed by autoradiography with Kodak X-OMAT film. A reaction devoid of DNA will be used as the negative control for each experiment.

Telomerase studies:

Selective sampling of small cell populations by microdissection: Biochemical assays attempting to find differences between normal and cancerous tissue, and between lesions at different stages of the disease in clinical material have often been hampered by contamination. This can be minimized by microdissecting tissue samples under the light microscope. Briefly, unstained, formalin-fixed 5-10 micron tissue sections are deparaffinized, rinsed in ethanol and 5% glycerol in water, and immediately processed under 100X magnification. Alternatively, 10-20 μm unstained cryostat sections are placed on glass slides, and thawed rapidly to 4°C. Areas of interest are selected on adjacent sections stained with hematoxylin and eosin. Specific cells of interest are identified and scraped away using a disposable 30-gauge needle, placing the sampled nests of cells directly into 10-20ml of the appropriate lysis buffer for nucleic acid or protein assays. Particular attention will be given to the sampling of histologically distinct tumor areas whenever feasible. We expect accrual rates of approximately 10-15 informative mastectomies and lumpectomies a month. In addition, a large repository of breast cancer cases are available in a frozen tissue bank in the department of pathology and in our laboratory.

Telomerase assay: The standard in vitro telomerase assay is based on a primer extension reaction in which telomerase synthesizes telomeric repeats onto oligonucleotide primers.

Two modifications in the protocol have decreased the number of cells necessary for detection from 10^7 to less than 10^2: a detergent lysis extraction which allows a more uniform recovery of telomerase activity at low cell numbers, and the PCR-based Telomeric Repeat Amplification Protocol (TRAP) in which the telomerase synthesized xtension products serve as templates for PCR amplification in the same reaction tube [14]. The commercially available HotStart 50^{TM} reaction tubes (Life Technologies Inc.) facilitate the two step assay by incorporating a wax barrier which remains intact during the telomerase phase of the assay, and melts during PCR.

A
Inv. CA 1
Inv. CA 2
Inv. CA 3
B
Whole section
Inv. CA
DCIS
+ control
C
Inv.CA
normal breast
FA-epithelium
FA-epithelium
FA-stroma
FA-stroma
Mucinous breast cancer
Poorly diff. breast cancer
2242-1A/5 (CIS)
2242-1A/5 (CA)
96-273/5 (FA-Epithelium)
96-273/5 (FA-Stroma)

Statement of Work:

Specific Aims 1 - 3: To develop molecular diagnostic techniques which may be used in the detection of breast cancer and to utilize these techniques in defining the genetic alterations associated with neoplastic progression in breast.

Task 1: Months 1-12: To develop a panel of informative microsatellite markers by examining a series of potential markers in DNA obtained from microdissected normal and breast cancer tissue.

Additional Task 1 undertaken: To determine the frequency of telomerase activation in breast cancer.

Status: Completed

Task 2: Months 12-24: To characterize the frequency of these genetic alterations in breast cancer by applying the microsatellite panel to a series of microdissected normal and breast cancer tissue.

Additional Task 2 to be undertaken: In the microdissected specimens in which loss of heterozygosity markers will be studied, we will perform telomerase assays on the same lysate, or on an adjacent section. The evaluation of both types of markers-microsatellite as well as telomerase on the same tissue will provide valuable information, and a direct comparison of the utility of these two markers.

CONCLUSIONS:

1) A panel of microsatellite and LOH markers have been selected for studying tumro progression in situ, in frozen sections of breast cancer.

2) While LOH was frequently observed in breast specimens, microsatellite instability was infrequent. Thus instability, unlike in other types of tumors, is not commonly observed in the panel we utilized.

3) Activation of telomerase occurs in the vast majority of breast cancers. Telomerase activation may occur early, raising the possibility that it is an early marker of breast cancer.

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Careful histologic confirmation and microdissection reveal telomerase activity in otherwise telomerase-negative breast cancers[1].

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Running title: Telomerase activity in telomerase-negative breast cancer

Key words: telomerase, TRAP assay, breast cancer, microdissection, biomarker

Abbreviations: TRAP: telomeric repeat amplification protocol, TLB:TRAP lysis buffer

Abstract

Studies of invasive breast cancers consistently identify a subset of tumors without telomerase activity, compromising its utility as a tumor marker. Telomerase-negative tumors may represent a biologically different subset or the result could be attributed to assay imperfections. To resolve this issue, we tested 105 invasive breast cancers for telomerase activity, and found that 23 (22%) tumors were telomerase-negative. Careful histologic confirmation of an adjacent cryosection and/or microdissection of pure tumor cells reduced this number to 5 (5%). Thus, truly telomerase-negative invasive breast cancers are rare, making this enzyme a potentially very useful tumor marker in breast cancer .

Introduction

Telomerase is a ribonucleoprotein enzyme active in germ cells, immortal cell lines, and the majority of malignancies examined, but inactive in most normal somatic tissues (1). It has been proposed as a potentially useful marker of malignancy. In several studies of primary invasive breast cancers, approximately 75-95% of tumors demonstrate telomerase activity, while 5-25% are telomerase-negative (2-7). Obviously, telomerase activity as a marker of invasive breast cancer is more useful if it detects 95% of the tumors rather than 75%, so the determination of the real telomerase-negative proportion of invasive breast cancers becomes crucial. Additionally, some studies (2, 3), although not all (4, 5), have found that smaller, lymph node-negative tumors are more likely to be telomerase-negative than their larger, lymph node-positive counterparts. Based upon this association between telomerase activity and traditional staging parameters, Hiyama et. al. (2) postulated that telomerase activity is acquired during tumor progression to metastasis. If this is true, then telomerase-positive tumors are biologically different (i.e. have the capability for metastasis) from their telomerase-negative counterparts, and this marker may be useful in clinical decision-making. An alternative explanation for telomerase-negative tumors is that a negative result is an artifact of the method of testing, and that the absence of telomerase activity in such tumors represents a false negative result. Possible reasons for a false negative result in the TRAP assay include insufficient viable invasive cancer cells examined, poor tissue maintenance, inadequate PCR amplification, or telomerase inhibitors in the surrounding tissue. To determine whether telomerase-

negative tumors are truly telomerase-negative or represent methodologic imperfections, we screened a series of breast cancers for telomerase activity. Breast cancer tissue specimens which tested negative for telomerase activity in the original screening subsequently underwent further evaluation using two methods: 1) histological confirmation, and 2) microdissection. We found that while 22% of invasive breast cancers were telomerase-negative in standard screening, only 5% were telomerase-negative if the sample tested for telomerase activity was carefully histologically confirmed and microdissected, suggesting that telomerase activity is in fact nearly ubiquitous in invasive breast cancers.

Methods

Samples:

The tissue samples used in this study were obtained from an existing frozen tumor bank in the Johns Hopkins Breast Cancer Research Program. The database for this tumor bank includes information regarding the gross tumor size and presence or absence of lymph node metastases in the breast cancers. No information regarding distant metastases is available, so formal staging was not performed. Only primary breast cancer specimens were considered for entry into the study. In addition, six specimens of normal breast tissue from reduction mammoplasties and mastectomy distant from the cancer site were included as negative controls. All tissue specimens were obtained from excess clinical specimens and institutional guidelines for the acquisition and maintenance of such specimens were followed.

Screening:

Breast tissue samples stored at -80°C in the Johns Hopkins Breast Cancer Program Tissue Inventory were tested for telomerase activity using the telomerase repeat amplification protocol (TRAP) (3). Prior to testing, the final pathology report from the paraffinized sections obtained at the time of the resection was reviewed for the breast tumors and confirmed as to the diagnosis of invasive breast cancer. For the original screening, a small quantity of tissue (approximately equal to 5-10 10μm sections) was shaved from the specimen into 60 μl of telomerase lysis buffer (1X TLB=0.5% (3-[(3-cholamidopropyl-

dimethyl-ammonio]-1-propane sulfonate), 10 mM Tris-HCl (pH 7.5), 1mM $MgCl_2$, 1mM EGTA, 5mM β-mercaptoethanol, 0.1mM (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochlorine and 10% glycerol) (3). The tissue samples were homogenized by physical disruption using the barrel of a 30-gauge needle, then left on ice for 30 minutes. The lysate was then centrifuged at 13,000g for 25 minutes at 4°C, and the supernatant was removed. Protein concentration was measured in each extract using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) and adjusted to 0.1μg/μl TLB. The extracts were flash frozen in liquid nitrogen and stored at -80°C until use. All extracts underwent TRAP assay within 4-6 weeks.

Histologic confirmation and microdissection:

All telomerase-negative breast cancers identified in the original screening were subjected to serial sectioning on a cryostat in order to obtain five-nine serial 10μm sections on five slides which were maintained at -80°C. One of these (from the middle section) was fixed, stained with hematoxylin and eosin (H&E), and evaluated by the study pathologist (GH). If a section was judged to have <10% invasive tumor or to exhibit necrosis, all the slides were discarded, and the tumor was resectioned until an acceptable area for testing was obtained. If an acceptable section was not obtained after three different areas of the gross tumor were cut, the banked tumor specimen was judged not to include viable invasive cancer. Once an acceptable area had been sectioned, the remaining four slides were divided, one was used for TRAP testing of the entire 10μm section, and the other three were used for microdissection of a pure tumor sample.

For the histologically confirmed testing, one 10μm cryosection of the frozen tissue sample was scraped off of the slide, homogenized with a 30-gauge needle, and lysed in 40 μl of TLB. For the testing of microdissected samples, an appropriate area of the tumor section was identified and marked on the H&E section by the study pathologist (GH). This area was judged to contain invasive tumor and to be free of

contaminating inflammatory cells or necrosis. Microdissection was performed on the adjacent frozen 10μm section. The slide was quick-thawed, and microdissection was immediately performed using a 30-gauge needle on a 1cc syringe under a dissecting microscope at 100x magnification similar to the manner previously described (8) (Figure 1). The microdissected sample was lysed in sufficient TLB in order to contain at least 20 tumor cells per μl of TLB. Preliminary examination of known telomerase-positive breast cancer samples revealed that telomerase activity could be reliably identified in microdissected samples containing at least 50 tumor cells (data not shown). For this reason, all microdissected samples contained at least 100 tumor cells in 5 μl TLB in order to be tested. The extraction was performed as described above and the supernatants were flash frozen and maintained at -80°C. All extracts underwent TRAP assay within 4-6 weeks.

TRAP assay

Each assay was run accompanied by an intraassay control where the tissue lysate was inactivated by heating to 94°C for 10 minutes or by the addition of 0.1μg RNase A and incubation at 37°C for 10 minutes. Each set of 20 assays included a negative control which contained the PCR reaction mixture only, no extract, as well as a positive control extract, containing 0.5μg of protein, from HBL-100 cells (American Type Culture Collection, Rockville, MD), an immortal cell line derived from human breast epithelium. This cell line has inherent telomerase activity. The one-tube telomerase assay was performed as described by Piatyszek and colleagues (9) with minor modifications. In brief, 5μl of the cell extract containing either 0.5μg of protein (whole sections) or the lysate from at least 100 tumor cells (microdissections) was added to a 50 μl reaction mixture containing 20mM Tris-Cl (pH8.3), 68mM KCl, 1.5mM $MgCl_2$, 1mM EGTA, 0.005% Tween-20, 50μM dNTP, 0.2μg TS oligonucleotide (5'-AATCCGTCGAGCAGAGTT-3') end-labeled with γ^{32}P-ATP via the T4 polynucleotide kinase reaction

(Operon), 5 ag of an Internal Telomerase Assay Standard (ITAS), and 2.5 U Taq DNA polymerase. The assay tubes additionally contained 0.1μg of CX oligonucleotide (5'-CCCTTACCCTTACCCTTACCCTAA-3') sequestered under a wax barrier by the HotStart method (Life Technologies, Gaithersburg, MD). The reaction mixtures were incubated at room temperature for 45 minutes, allowing the telomerase enzyme to extend the TS substrate. The reaction mixture was then heated to 94°C for 5 minutes followed by 30 cycles of PCR at 56°C for 30 seconds, 72°C for 45 seconds, 94°C for 30 seconds. The PCR products were electrophoresed on a 10%, nondenaturing, 0.5mm polyacrylamide gel (18 x 16 cm) run in 0.5x TBE buffer at 350 volts until the bromophenol blue marker dye had run off. The gels were dried and exposed overnight to Kodak X-OMAT film (Eastman Kodak, Rochester, NY). Results were scored in a binary fashion, with a positive result defined as any banding pattern (laddering) beyond background, and a negative result as no banding beyond background with a positive ITAS control sequence. If the ITAS control sequence was absent in a lane with no banding, the PCR reaction was repeated following phenol and chloroform extraction of the enzyme assay. All negative results were repeated for confirmation when possible.

Statistical analysis

The proportion of tumors exhibiting telomerase activity was compared to tumor size (greater than versus less than or equal to 2 cm) and lymph node status (metastases present or absent) using two-tailed Fisher's exact test with significance level $p \leq 0.05$.

Results

Screening

Twenty-eight (27%) of the 105 tumors tested were negative initially for telomerase activity by the TRAP assay. Ten of these showed no internal control (ITAS) band suggesting the presence of PCR inhibitors in the reaction. Following phenol/chloroform extraction, ITAS was positive in all ten tumors; five showed positive telomerase banding pattern, while five remained negative for telomerase. Thus 23 (22%) of the 105 tumors lacked detectable telomerase activity. The proportion of tumors exhibiting telomerase activity did not differ significantly by gross tumor size or lymph node status (Table 1). Six normal breast tissue samples obtained from reduction mammoplasty (5 specimens) and 14 mastectomy samples distant from the primary site (4 specimens) were uniformly negative for telomerase activity (data not shown). Each section was histologically confirmed to contain at least 5% breast epithelium.

Histologic confirmation

The TRAP assay typically involves very small quantities of tissue extract for two reasons - its sensitivity and the tendency for tissues to contain inhibitors of the PCR amplification step. The purpose of the histologically confirmed method of testing is to directly assure that an adequate proportion of the tissue being tested in fact contained viable, nonnecrotic, invasive breast cancer cells, an important step given the gross heterogeneity of breast cancers. One hundred and five tumors were screened. In all cases, the pathologic report of the paraffinized tumor sample from the original resection confirmed the diagnosis of invasive breast cancer. All 23 telomerase-negative tumors identified in the original screening effort underwent sectioning with histologic confirmation of the adjacent 10μm section (Figure 2). Seven (30%) did not have sufficient invasive tumor in the initial sectioning to be tested (1 necrotic, 2 in situ carcinoma only, 4 without notable tumor cells at all). Further sectioning of the tumor mass provided an adequate sample for testing in three tumors. However, in four tumors no adequate tumor sample was ever obtained despite three separate sectioning efforts (1 necrotic tumor, 2 residual *in situ* carcinoma only, 1 without any residual cancer). Once an adequate section containing more than 10% viable invasive carcinoma was

obtained in the 19 remaining tumors, TRAP analysis of the entire section was performed. Thirteen (68%) were telomerase-positive (Figure 3, left panel), six (32%) were telomerase-negative (Figure 3, middle and right panels) in repeated assays. Using this technique to improve the quality of the tissue being tested for telomerase activity, only 6 (6%) of 105 breast cancers lacked detectable telomerase activity (Figure 2).

Microdissection

Breast tumors often include normal stroma, *in situ* carcinoma, and fibrosis from previous manipulation such as biopsy. The purpose of the microdissection method of testing is to test a pure sample of tumor cells absent stroma, inflammation, or other contaminating cells. The six tumor sections which remained telomerase-negative after histologic confirmation were microdissected and a pure tumor sample was tested. One was telomerase-positive (Figure 3, middle panel), while five remained telomerase-negative (Figure 3, right panel). Of the five tumors which remained persistently telomerase-negative despite careful histologic confirmation and microdissection, two were smaller than 2 cm, two were larger, and one was inflammatory. Two were from patients without axillary lymph node involvement, three were from patients with involved nodes. Thus, of the 105 tumors screened, the addition of this technique decreased the proportion of telomerase-negative tumors to 5% (Figure 2).

As a control for the technique of microdissection, nine of the tumors which tested positive after histologic confirmation were microdissected and the pure tumor sample was tested. All remained telomerase-positive (data not shown).

Tumor heterogeneity

When possible, a second set of sections from a different region of the 19 screened telomerase-negative tumors was cut and tested for telomerase activity using the same methods described above. In this way, a separate section of viable invasive carcinoma from another area of the tumor bulk was tested in 15 of the tumors. In 11 of these 15 tumors, the second area gave similar results to the previous telomerase assay. Four tumors, however, demonstrated telomerase activity heterogeneity in that the second facet of the tumor mass gave a reproducibly different result from the initial facet tested. As before, a telomerase-negative result was not considered truly negative unless the microdissected sample was tested

twice without evidence of telomerase activity. One tumor which demonstrated telomerase activity heterogeneity after careful histologic confirmation was found to be telomerase-positive consistently after microdissection, suggesting that the difference between the two facets of the tumor related to stromal differences, perhaps due to the presence of telomerase inhibitors. The other three tumors remained heterogeneous at the level of the tumor cells, with a telomerase-positive region and a telomerase-negative region despite microdissection.

Discussion

In this study, we have tried to define the true telomerase-negative proportion of invasive breast cancers. This definition carries both biological and clinical implications. If there exists a subset of invasive breast cancers which lack telomerase activity, an obvious question is whether such cancers are inherently less aggressive than their telomerase-positive counterparts. Clinical support for this concept comes from those studies (2) which find a correlation between breast cancer stage and likelihood of exhibiting telomerase activity, with higher stage tumors more likely to be telomerase-positive. Conversely, if the true telomerase-negative rate of invasive breast cancers is very low, this tumor marker gains greatly in its potential clinical applications, particularly since in this as well as in other studies, the specificity of the TRAP assay is very high. According to recent studies, virtually no normal breast tissue or benign breast lesion exhibits telomerase activity with the possible exception of a proportion of fibroadenomas (2-6).

Previous studies of invasive breast cancers have found a telomerase-positive rate of between 73% (5) and 95% (6). Our screening efforts detected telomerase activity in 78%, which is consistent with these and other studies. Unlike Hiyama et al. (2), and Sugino et al. (5), but in concordance with findings of Nawaz et al (4) we did not detect a correlation between telomerase activity and clinical stage at the time of screening. In fact, the five tumors which remained telomerase-negative at the end of the study were of mixed size and lymph node status. Telomerase activity was examined as a continuous variable in Dr. Sugino's study, which was hampered by the use of an older, less quantitative assay technique than that which is currently available. In addition, these investigators did not use an internal control sequence to standardize for the efficiency of the PCR reaction, which may also have confounded their results. Dr.

Hiyama's study, however, examined telomerase activity in much the same manner as our own study. Their study was slightly larger (140 invasive breast cancers) than ours (105 invasive cancers) and included a greater proportion of smaller, lymph node-negative tumors. However, this study was sufficiently powered to reliably detect an association of tumor size or lymph node status and telomerase activity. The discrepant result may reflects the potential for an excess of normal tissue in the banked sample from smaller tumors, which are more likely to be from patients who are lymph node-negative.

After careful histologic confirmation by H&E staining of the immediately adjacent section, the proportion of telomerase-negative breast cancers fell to 6%. Thus, by this simple maneuver, the sensitivity of the TRAP assay in detecting telomerase activity rose markedly, making telomerase activity a considerably more useful tumor marker. This finding also raises justifiable concern about the use of the official pathology reports to define banked tumor samples. Breast cancers in particular are a heterogeneous group both macroscopically and microscopically. Macroscopically, the palpated mass can include fibrosis, inflammation, and post-biopsy changes as well as cancer. Microscopically, the tumor typically contains varying amounts of inflammatory cells, stroma, normal ductal tissue, preneoplastic tissue, and *in situ* carcinoma as well as invasive cancer.

Further evaluation of the six persistently telomerase-negative tumors with microdissection by analysis of pure tumor samples following microdissection detected telomerase activity in one sample. Thus, the proportion of invasive breast cancers without telomerase activity was found in this study to be 5%. Microdissection and TRAP analysis of one of four telomerase-negative portions of the telomerase-heterogeneous tumors also detected telomerase activity in the pure tumor cell sample. There are several potential reasons for a histologically confirmed telomerase-negative tumor to be telomerase-positive when a microdissected pure tumor sample is tested. Stromal dilution of the telomerase-positive tumor cells is possible but unlikely given the sensitivity of the TRAP assay and the fact, that to be eligible, the section had to contain at least 10% tumor cells. Although the detection of the ITAS internal control sequence makes the presence of Taq inhibitors unlikely, telomerase inhibitors in the surrounding tissue could dampen telomerase activity of the tumor cells while allowing the ITAS control sequence to be amplified

normally. Such inhibitors, which have been demonstrated in breast cancer tissues (10), would be unlikely in a pure tumor population.

The reason for the absence of telomerase activity in the tumors which remained persistently telomerase-negative is unclear. Evaluation of the size and lymph node status of the tumor is notable for the variety of stages represented, arguing against a link with prognosis, although without outcome data this is impossible to truly assess. It is possible that the tissue was mishandled at the time of collection, for example by leaving the specimen under hot lamps for an excessive amount of time at the time of tumor banking or inadvertent fixation. Regardless of cause, these tumors appear to represent a very small fraction of the total population of invasive breast cancers.

Heterogeneity in telomerase activity was found in three breast cancers of the 15 tumors with two different areas examined. These tumors had areas which were reproducibly telomerase-positive and other areas which were telomerase-negative even in microdissected pure tumor cell samples. This suggests that telomerase activity, like genomic instability, can be heterogeneous from one area of the invasive cancer to another, and could be acquired during tumor progression.

This study demonstrates that telomerase activity is nearly ubiquitous in invasive breast cancers. Based on this finding, the previous studies mentioned earlier in which telomerase activity was absent from a substantial proportion of tumors were likely confounded by gross or microscopic tumor heterogeneity producing a false negative result in the telomerase activity assay. Future investigators, especially those working in breast cancer, should be skeptical regarding the content of banked tumors and confirm all samples by staining of an adjacent section or similar method of direct histologic confirmation. In this study, this technique alone raised the percentage of telomerase-positive tumors to 95%. Microdissection, while identifying the true telomerase-positive nature of a few samples more, is too tedious and contributes too little to be used routinely. It does, however, allow testing of a pure tumor sample and would also be useful in studies of a progression model for the activation of telomerase within tumors and to dissociate the ability of neighboring stromal cells to inhibit telomerase activity.

Based upon the results of this study, which suggest that telomerase activity is present and readily measurable in the vast majority of invasive breast cancers, future directions of exploration might involve the use of this tumor marker as a diagnostic tool. For instance, in the future, telomerase activity in cells extruded in nipple aspiration fluid could provide a valuable adjunct to mammography, especially in young women. In addition, our data imply that exploration of telomerase-positivity or -negativity as a predictor of response or outcome is not likely to be fruitful, although quantitative examination of this marker may in fact prove useful.

Table 1. Comparison of presence or absence of telomerase activity with tumor size and lymph node metastasis in the screened breast cancers.

	No.	Telomerase-positive no. (%)	Telomerase-negative no. (% ± SE)
Tumor size, cm			
≤ 2	22	20 (91)	2 (9 ± 12)
> 2	80	60 (75)	20 (25 ± 10)
unknown	3	2 (67)	1 (33)
Lymph node metastases			
absent	31	24 (77)	7 (23 ± 15)
present	51	37 (72)	14 (27 ± 12)
unknown	23	21 (91)	2 (9)

Figure 1. Photomicrographs demonstrating the technique of microdissection. (A) Invasive tumor before microdissection, (B) an adjacent cryosection of the same tumor after microdissection demonstrating the removal of a pure population of tumor cells.

Figure 2. Flow diagram depicting the results of histologic confirmation, microdissection, and subsequent TRAP analysis of banked breast cancers which were telomerase-negative on original screening.

Figure 3. Left panel: Telomerase activity in one tumor which was telomerase-negative in the original screening (1A) but was telomerase-positive in the histologically confirmed whole section (1B). In this as in all telomerase-positive sections, the microdissected pure tumor sample also remained telomerase-positive (1C).

Middle panel: Telomerase activity in one tumor which was telomerase-negative in the original screening (not shown), was very weakly telomerase-positive in a histologically confirmed whole section (2B) but was clearly telomerase-positive following microdissection (2C).

Right panel: Telomerase activity in one tumor which was telomerase-negative in the original screening (not shown) and remained telomerase-negative in spite of histologic confirmation (3B) and microdissection (3C). Cell lysates of HBL100 breast epithelial cells (0.5 μg protein) served as a positive control, while the negative control reaction contained an equal volume of lysis buffer in lieu of tissue lysate. Inactivation of the telomerase activity in the extracts by heating the reaction to 95^0C for 10 min is indicated by + on top of the lanes. The amplification products of 10 ag of the internal TRAP assay standard (ITAS) is shown.

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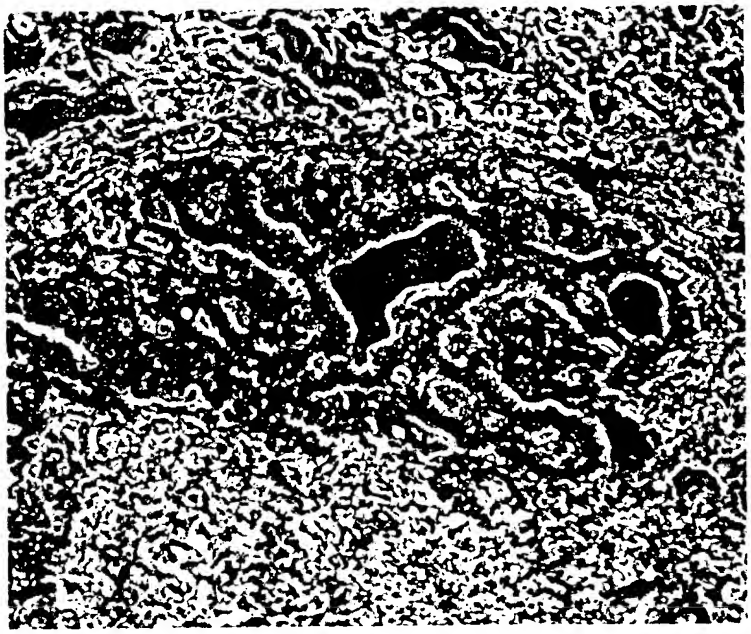
A



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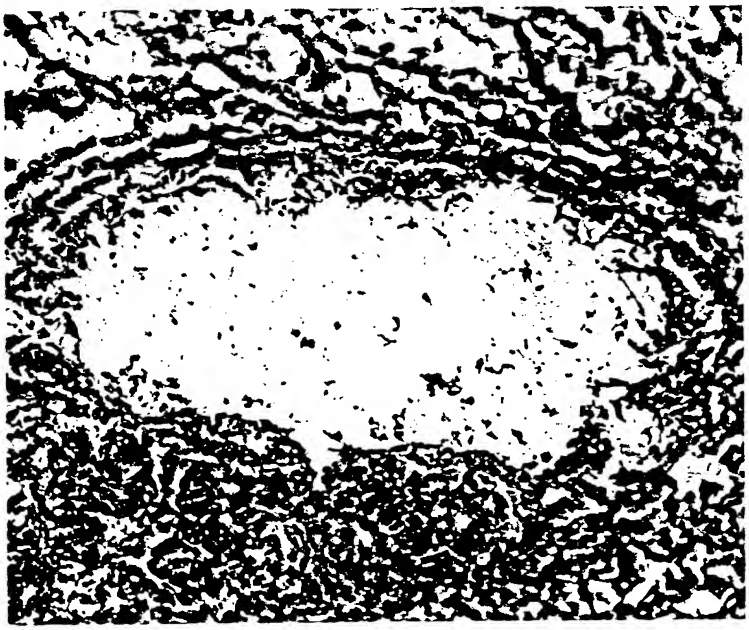
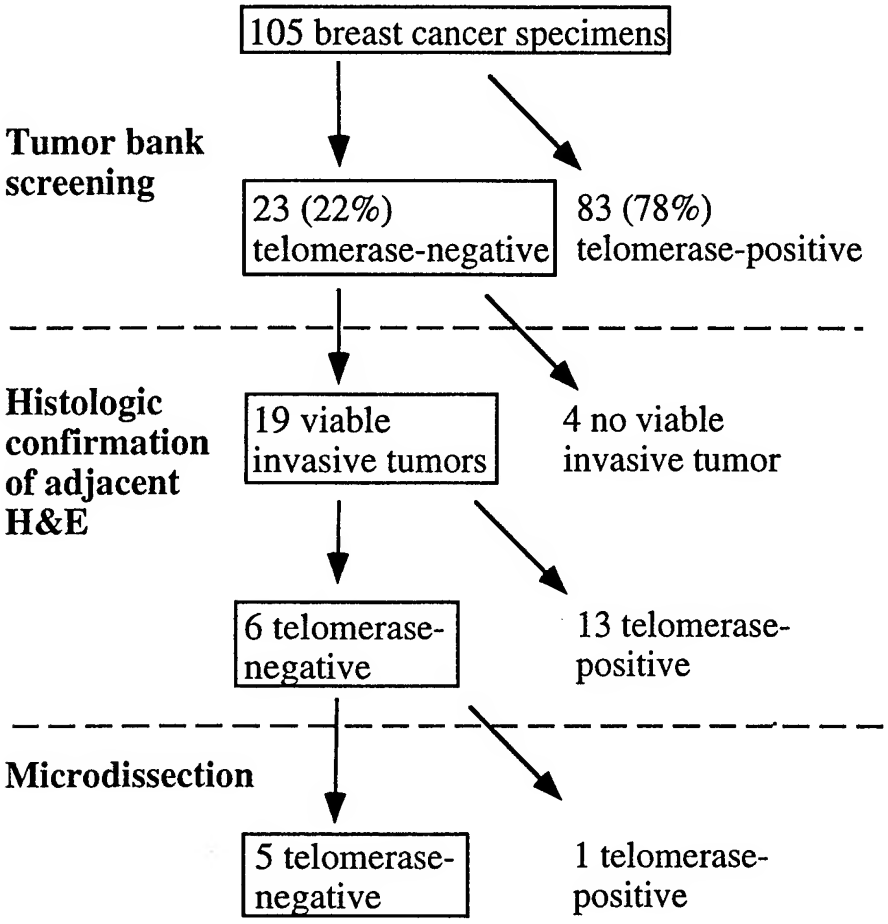
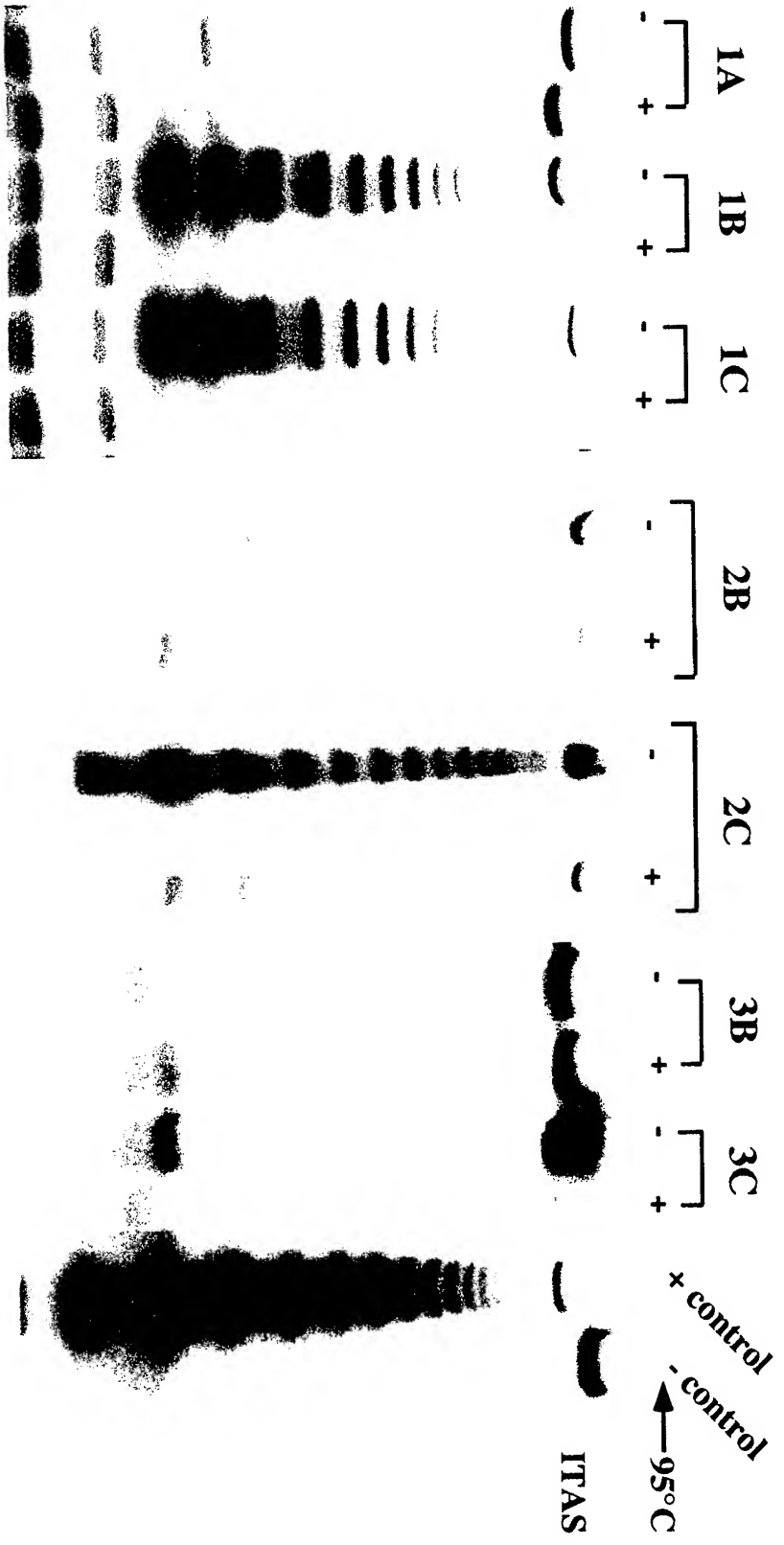


Figure 1


105 breast cancer specimens
Tumor bank screening
23 (22%) telomerase-negative
83 (78%) telomerase-positive
Histologic confirmation of adjacent H&E
19 viable invasive tumors
4 no viable invasive tumor
6 telomerase-negative
13 telomerase-positive
Microdissection
5 telomerase-negative
1 telomerase-positive


1A
1B
1C
2B
2C
3B
3C
+ control
- control
95°C
ITAS